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Latent Membrane Protein 1 (LMP1) and LMP2A Collaborate To Promote Epstein-Barr Virus-Induced B Cell Lymphomas in a Cord Blood-Humanized Mouse Model but Are Not Essential

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ABSTRACT Epstein-Barr virus (EBV) infection is associated with B cell lymphomas in humans. The ability of EBV to convert human B cells into long-lived lymphoblastoid cell lines (LCLs) *in vitro* requires the collaborative effects of EBNA2 (which hijacks Notch signaling), latent membrane protein 1 (LMP1) (which mimics CD40 signaling), and EBV-encoded nuclear antigen 3A (EBNA3A) and EBNA3C (which inhibit oncogene-induced senescence and apoptosis). However, we recently showed that an LMP1-deleted EBV mutant induces B cell lymphomas in a newly developed cord blood-humanized mouse model that allows EBV-infected B cells to interact with CD4 T cells (the major source of CD40 ligand). Here we examined whether the EBV LMP2A protein, which mimics constitutively active B cell receptor signaling, is required for EBV-induced lymphomas in this model. We find that the deletion of LMP2A delays the onset of EBV-induced lymphomas but does not affect the tumor phenotype or the number of tumors. The simultaneous deletion of both LMP1 and LMP2A results in fewer tumors and a further delay in tumor onset. Nevertheless, the LMP1/LMP2A double mutant induces lymphomas in approximately half of the infected animals. These results indicate that neither LMP1 nor LMP2A is absolutely essential for the ability of EBV to induce B cell lymphomas in the cord blood-humanized mouse model, although the simultaneous loss of both LMP1 and LMP2A decreases the proportion of animals developing tumors and increases the time to tumor onset. Thus, the expression of either LMP1 or LMP2A may be sufficient to promote early-onset EBV-induced tumors in this model.

IMPORTANCE EBV causes human lymphomas, but few models are available for dissecting how EBV causes lymphomas *in vivo* in the context of a host immune response. We recently used a newly developed cord blood-humanized mouse model to show that EBV can cooperate with human CD4 T cells to cause B cell lymphomas even when a major viral transforming protein, LMP1, is deleted. Here we examined whether the EBV protein LMP2A, which mimics B cell receptor signaling, is required for EBV-induced lymphomas in this model. We find that the deletion of LMP2A alone has little effect on the ability of EBV to cause lymphomas but delays tumor onset. The deletion of both LMP1 and LMP2A results in a smaller number of lymphomas in infected animals, with an even more delayed time to tumor onset. These results suggest that LMP1 and LMP2A collaborate to promote early-onset lymphomas in this model, but neither protein is absolutely essential.

KEYWORDS EBV, Epstein-Barr virus, LMP1, LMP2A, humanized mouse, lymphoma

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Epstein-Barr virus (EBV) is a human herpesvirus that contributes to a variety of human B cell lymphomas, including lymphoproliferative disease (LPD) in immunocompromised hosts, diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma (BL), and Hodgkin lymphoma (HL) (1, 2). EBV-infected tumors are composed largely of latently infected cells, in which the virus persists as a nuclear episome and is replicated by the host cell DNA polymerase (3). Several different forms of viral latency, which differ in regard to the number of viral genes expressed, can occur in EBV-positive human lymphomas (3). However, only type III EBV latency can convert primary B cells *in vitro* into long-term lymphoblastoid cell lines (LCLs). Nevertheless, this form of latency, which allows the expression of each of the nine viral latency proteins (plus the small EBV-encoded nuclear RNAs [EBERs] and virally encoded microRNAs), is also the most immunogenic form and thus is usually restricted to tumors of immunosuppressed patients.

The generation of EBV-transformed LCLs *in vitro* requires both EBV-encoded nuclear antigens (EBNAs), including EBNA1, EBNA2, EBNA3A, and EBNA3C, and latent membrane protein 1 (LMP1) (3). The cellular gene expression pattern in EBV-driven LCLs largely reflects the transcriptional effects of the EBNA2 and LMP1 proteins (4). EBNA2 interacts directly with the cellular protein RBP-Jκ (CBF1) to mimic the effect of constitutive Notch signaling and promote B cell proliferation (5, 6). EBNA2 (directly or indirectly) activates the expression of c-Myc, cyclin D2, and E2F1 in B cells, and c-Myc expression is required for the proliferation of LCLs (7, 8). LMP1 mimics the effect of constitutively active CD40 signaling, thereby activating the NF-κB, AP1, and ATF2 transcription factors and inhibiting apoptosis (9–12).

Although the establishment of long-term LCLs *in vitro* requires LMP1 expression, the rapid proliferation of B cells during the first week of EBV infection *in vitro* is driven largely by EBNA2 (13). During this initial "proliferative" period, EBV-infected cells replicate more rapidly (dividing every 12 h) than at later times (dividing every 24 h) and do not express appreciable amounts of LMP1 or NF-κB (13). Thus, EBNA2 can drive B cell proliferation in the absence of LMP1. The EBNA3A and EBNA3C proteins, which collaboratively turn off the expression of the tumor suppressor protein p16 (14, 15) and the proapoptotic protein BIM1 (16, 17), are also required for long-term LCL outgrowth, as is EBNA1, which mediates the replication of the latent viral genome (3).

Another EBV-encoded protein, LMP2A, could potentially be required for EBV-induced lymphomas in humans, even though it is largely dispensable for EBV-induced B cell transformation *in vitro*. LMP2A mimics the effect of low-level constitutive B cell receptor (BCR) signaling (18–22), and EBV-negative B cell lymphomas often have cellular mutations or other abnormalities that lead to constitutive BCR signaling (23, 24). Furthermore, in transgenic mouse models, LMP2A promotes the survival of B cells in the periphery that have not undergone a productive BCR rearrangement (21) and cooperates with c-Myc to induce Burkitt-like B cell lymphomas (25). LMP2A is also required for the ability of EBV to transform germinal center (GC)-derived B cells *in vitro* that have not undergone a productive BCR rearrangement (26).

Although EBV efficiently infects many different types of B cells *in vitro* and *in vivo*, including naive B cells, GC B cells, and post-germinal center memory B cells (3, 27, 28), long-term latent EBV infection appears to be restricted to the memory B cell compartment in immunocompetent humans (3, 29). One model to explain this phenomenon proposes that EBV infection of naive B cells promotes a GC-like reaction (via the effects of LMP1 and LMP2A) that converts naive B cells into memory-like B cells even in the absence of a true (i.e., helper T cell-dependent) GC reaction (3, 27). Another model is based upon *in vitro* studies showing that EBV infection of naive B cells induces T cell-independent somatic hypermutation (SHM) (but not class switching) by inducing the expression of activation-induced cytosine deaminase (AID) (27). This model proposes that EBV-infected B cells that have undergone GC-independent SHM have a selective survival advantage *in vitro* and *in vivo* (27). Nevertheless, a subset of EBV-positive posttransplant lymphoproliferative disease (PTLD) lesions are derived from naive B cells (30), indicating that EBV can also successfully establish long-term latent

infection in naive B cells of immunocompromised hosts. In addition to preferentially establishing latency in long-lived memory B cells, another potential mechanism by which EBV may ensure the prolonged survival of latently infected B cells is to inhibit terminal plasma cell differentiation (31–33).

Animal and cell models that can examine how EBV infection affects different aspects of normal B cell biology have been difficult to develop and often give contradictory results. For example, while LMP2A expression in transgenic mice has been reported to promote autoimmunity and plasma cell differentiation (34, 35), and LMP1 expression can lead to lymphomas (11, 36), the combination of LMP1 and LMP2A together in transgenic mice normalizes the phenotypes induced by the expression of LMP1 and LMP2A alone (37). Thus, the effects of LMP1 and LMP2A on EBV pathogenesis and B cell biology may be most biologically relevant when studied in the context of the intact virus using models that allow B cells and T cells to interact.

Here we have used a newly developed cord blood-humanized mouse model to examine the effects of the loss of LMP2A (in the presence and absence of the concomitant loss of LMP1) on EBV-induced lymphoma formation and to ask whether EBV infection of naive (cord blood-derived) B cells regulates their ability to undergo plasma cell differentiation. Using this model, we recently showed that LMP1 is unexpectedly dispensable for EBV-induced lymphoma formation, since CD40L-expressing human CD4 T cells can partially substitute for LMP1 (38). Our results here suggest that neither LMP1 nor LMP2A is absolutely required for EBV-induced lymphomas in this model, even when both proteins are simultaneously deleted. Nevertheless, the deletion of LMP2A alone delays the onset of lymphomas, and the deletion of both LMP1 and LMP2A together reduces the number of EBV-infected animals that develop lymphomas and further delays the onset of tumor development. Furthermore, we find that EBV infection strongly inhibits plasma cell differentiation in this model and show that this effect does not require the expression of either LMP1 or LMP2A. Together, these findings suggest that the cord blood-humanized mouse model may be useful for dissecting how EBV proteins (including the nuclear EBNA proteins) and/or virally encoded small RNAs contribute to EBV-induced lymphomas in humans and for understanding how EBV infection regulates B cell biology and differentiation.

RESULTS

B cells infected with the LMP2A-KO and LMP1/LMP2A-KO viruses stably engraft and induce invasive B cell lymphomas in cord blood-humanized NSG mice. We used a cord blood-humanized mouse model to determine if LMP2A is required for EBV-induced lymphomas in the presence, or absence, of LMP1 expression. CD34-depleted human cord blood cells were either mock infected or infected for 1.5 h *in vitro* with 10,000 infectious particles (green Raji cell units [GRUs]) of wild-type (WT) EBV (B95.8 strain bacmid), an EBV mutant (B95.8 strain) deleted for the LMP2A gene (LMP2A-KO), or an EBV mutant (B95.8 strain) deleted for both the LMP2A and LMP1 genes (LMP1/2A-KO) and then injected intraperitoneally (i.p.) into NSG (NOD/LtSz-*scid/IL2R*null) mice. We previously showed that almost all WT EBV-infected mice in this model develop activated DLBCLs with type III EBV latency, which often invade the pancreas, liver, and gallbladder (38, 39). In contrast, mice injected with uninfected cord blood consistently engraft human T cells, but only a portion of mice sustain long-term coengraftment of human B cells, and they do not develop B cell lymphomas.

As shown in Fig. 1A, as expected, almost all mice injected with WT EBV-infected cord blood developed tumors, whereas injection of uninfected cord blood did not result in any tumors. Mice injected with LMP2A-KO virus-infected cord blood cells developed lymphomas at a frequency similar to that for WT virus-infected animals (Fig. 1A), although the tumors derived from this mutant often occurred at a later time point than did the WT virus-induced tumors (Fig. 1B). Somewhat surprisingly, a substantial subset of mice (47%) infected with an EBV mutant missing both LMP2A and LMP1 (LMP1/2A-KO virus) also developed lymphomas, although the number of animals developing lymphomas was significantly smaller than the number of animals developing lympho-

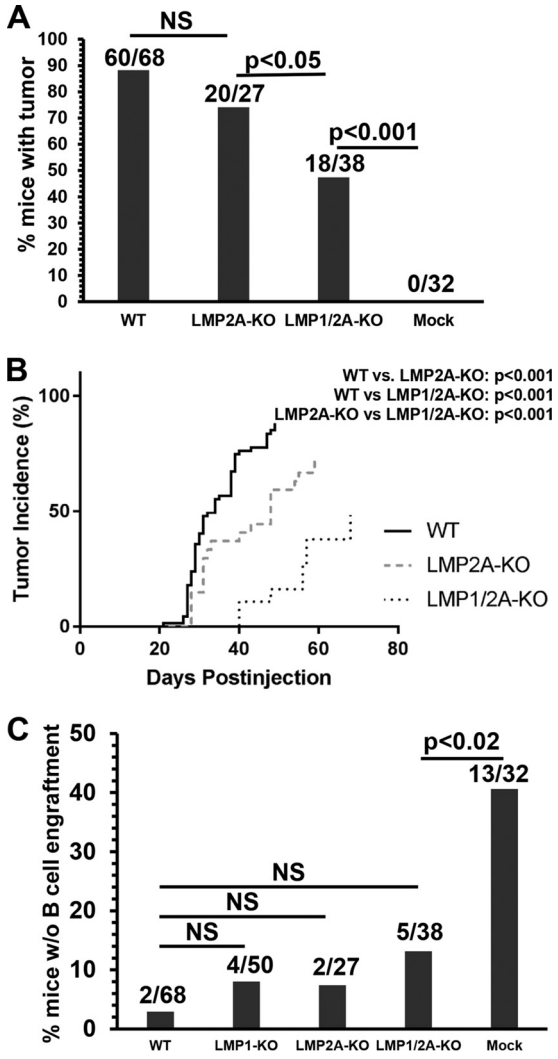

FIG 1 Both the LMP2A-KO and LMP1/2A-KO viruses can induce invasive B cell lymphomas in cord blood-humanized NSG mice. (A) Percentages of NSG mice injected with WT, LMP2A-KO, and LMP1/2A-KO viruses and mock-infected animals developing lymphoma tumors. (B) Comparisons of times to tumor incidence for animals infected with the WT, LMP2A-deleted, or LMP1/2-deleted virus. (C) Proportions of WT, LMP2A-KO, and LMP1/2A-KO virus-infected and mock-infected mice without B cell engraftment (assessed by CD20 IHC at days 50 to 60 after cord blood injection). The number of mice with no B cell engraftment relative to the total number of mice used in each group is indicated; *P* values were calculated by using a two-tailed Fisher exact test. NS, not significant.

mas following infection with the WT or LMP2A-KO virus (Fig. 1A). Tumors derived from the LMP1/2A-KO double mutant virus also occurred at later time points than did tumors derived from the LMP2A mutant or the WT virus (Fig. 1B). Animals injected with either LMP2A-KO virus-infected or LMP1/2A-KO virus-infected cord blood were also substantially more likely to sustain long-term B cell engraftment (assessed by CD20 immunohistochemistry [IHC] at days 50 to 60 post-cord blood injection) than were mice injected with uninfected B cells (Fig. 1C).

To confirm that the LMP1/2A-KO virus used in these studies is profoundly defective for transforming B cells *in vitro*, the outgrowths of peripheral B cells infected with two different amounts of the WT, LMP2A-KO, or LMP1/2A-KO virus were compared. Immunoblot analysis of infected B cells *in vitro* confirmed that WT virus-infected cells, but not LMP2A-KO or LMP1/2A-KO virus-infected cells, expressed LMP2A and that WT virus- and LMP2A-KO virus-infected cells, but not LMP1/2A-KO virus-infected cells, expressed LMP1 (data not shown). As expected, the LMP1/2A-KO virus was unable to produce

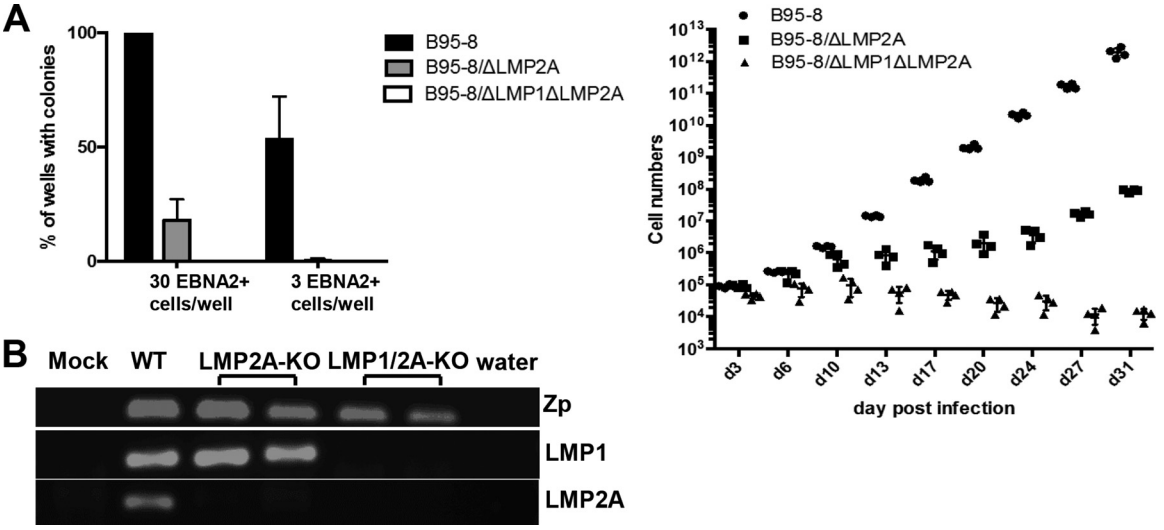

FIG 2 The LMP1/2A-KO mutant is defective for transformation *in vitro* but induces lymphomas in cord blood-humanized mice *in vivo*. (A, left) Purified B cells were infected with WT EBV (B95.8 strain), the LMP2A-KO virus, or the LMP1/2A-KO virus. Three days after infection, cells were immunostained for EBNA2 protein expression. Infected cells were plated at a concentration of 3 or 30 EBNA2-positive cells per well in a 96-well cluster plate coated with Wi-38 feeder cells. The bar graph shows the percentages of outgrown cells 28 days after infection of 4 independent blood samples. Error bars represent the means with standard deviations. (Right) A total of 10^5 B cells were exposed to supernatants containing WT EBV (B95.8 strain), the LMP2A-KO virus, or the LMP1/2A-KO virus at a multiplicity of infection of 20 EBV genome equivalents per cell. The number of B cells generated by infection was recorded every 3 or 4 days until day 31 postinfection. The dot plot shows the results of infection of 4 independent B cell samples. (B) PCR analysis was performed on isolated tumor DNA prepared from 1 mock-infected spleen, 1 WT EBV-infected tumor, 2 different LMP2A-KO virus-infected tumors, 2 LMP1/2A-KO virus-infected tumors, or a water control, using primers that amplify the LMP1 gene, the LMP2A gene, or the viral BZLF1 promoter, as indicated.

long-term LCLs, and cells infected with this virus had much decreased proliferation compared to that of WT virus-infected cells (Fig. 2A). Interestingly, the LMP2A-KO virus was also partially defective for inducing B cell proliferation and transformation *in vitro* in comparison to equal titers of the WT virus (Fig. 2A).

To demonstrate that tumors obtained *in vivo* with the LMP2A-KO and LMP1/2A-KO viruses were not contaminated with the WT virus, tumor DNA was isolated from paraffin-fixed slides, and PCR analysis was used to determine if either the LMP1 or LMP2A gene was present (Fig. 2B). These results showed that no detectable WT virus contaminated the tumors derived from the mutant viruses. Thus, neither LMP1 nor LMP2A is absolutely essential for the ability of EBV-infected B cells to stably engraft, or cause invasive lymphomas, in the cord blood-humanized mouse model.

Comparison of the phenotypes of lymphomas induced by the WT EBV, LMP2A-KO, and LMP1/2A-KO viruses. To determine if the loss of either LMP2A alone or LMP1 and LMP2A together affects the phenotype of EBV-induced lymphomas in the cord blood-humanized mouse model, tumors were stained with hematoxylin and eosin (H&E) as well as a variety of different antibodies to assess the B cell differentiation state and viral expression pattern. Results are summarized in Table 1. All of the tumors obtained with the WT virus or the LMP2A mutant virus had aggressive DLBCL-like lymphomas, showing diffuse sheets of large, atypical lymphoid cells with readily apparent mitotic figures (Fig. 3). In contrast, while many of the lymphomas derived from the LMP1/2A-KO mutant also appeared similar to aggressive DLBCLs, some of the lesions derived from this mutant were more similar to polymorphic PTLD lesions, displaying a mixture of large- and medium-sized atypical lymphoid cells interspersed with plasma cells (Fig. 3, bottom right, and Table 1). The majority of tumors were intraparenchymal in the pancreas, often with extension into adjacent liver tissue and forming masses in the mesentery/mesothelium. Some tumors also involved the white pulp of the spleen and perivascular areas of the lung.

The WT EBV-infected, LMP2A-KO virus-infected, and LMP1/2A-KO virus-infected tumors each expressed CD20 (confirming B cell identity) and the EBV latency protein

TABLE 1 Summary of WT, LMP2A-KO, and LMP1/2A-KO tumor morphologies[a]

Virus	Mouse	Morphology	% of IRF4$^+$ CD20$^+$ cells	Frequency of IRF4$^+$ CD20$^-$ cells	No. of mitotic figures/ high-power field
WT	1	DLBCL	80	Occasional	1–2
	2	DLBCL with some immunoblastic morphology	25	Rare	2–3
	3	DLBCL	80	Occasional	2–3
	4	DLBCL	Variable, up to 100	Rare	2–3
	5	DLBCL	NA	NA	4–5
	6	DLBCL	80	Occasional	2–3
	7	DLBCL	NA	NA	4–5
LMP2A-KO	1	DLBCL with scattered plasmacytoid cells	80	Rare	2–3
	2	DLBCL	50–80	Rare	1–2
	3	DLBCL	50–80	Rare	2–3
	4	DLBCL	50–80	Rare	3–4
	5	DLBCL	NA	NA	NA
	6	DLBCL	30	Rare	3–4
	7	DLBCL	30	Rare	4–5
LMP1/2A-KO	1	DLBCL	$<$20	Occasional	4–6
	2	DLBCL	20–50	Occasional	4–5
	3	DLBCL	20	Occasional	2–3
	4	DLBCL	20–50	Occasional	2–3
	5	DLBCL	NA	NA	NA
		Polymorphous PTLD lesions	20–50	Occasional	2–3
	6	DLBCL (pancreas)	20–30	Occasional	2–3
		Polymorphous PTLD lesions (bile ducts)	50	Occasional	2–3
	7	DLBCL (liver)	10	Rare	2–3
		Polymorphous PTLD lesions (pancreas)	50	Occasional	2–3

[a]NA, not available due to poor tissue fixation.

EBNA2 (confirming type III latency) (Fig. 4A and B). All tumors (regardless of whether they were infected with WT EBV or mutant viruses) also expressed interferon regulatory factor 4 (IRF4) (Fig. 4A), a marker for early plasma cell differentiation and a surrogate immunohistochemical marker for the "activated B cell type" of DLBCLs (40, 41). IRF4 is also an essential survival factor for activated B cell lymphomas in humans (41) and for LCLs *in vitro* (42, 43). In contrast to lymphoma cells, T cells infiltrating the tumors had very little IRF4 expression (Fig. 4A). WT and LMP2A-KO virus-infected lymphomas also expressed similar levels of LMP1 (Fig. 4B and C).

LMP2A-KO and LMP1/2A-KO virus-induced lymphomas have enhanced c-Myc expression and decreased BIM1 expression. Since EBNA2 activates the expression of the potent cellular oncogene c-Myc in LCLs *in vitro*, we next examined whether c-Myc is expressed in WT or mutant virus-infected DLBCLs in the cord blood-humanized mouse model. As shown in Fig. 5 (left), high c-Myc levels were found in a subset WT, LMP2A-KO, and LMP1/2A-KO virus-infected lymphoma cells. Given the ability of EBNA3A and EBNA3C to inhibit the expression of the proapoptotic protein BIM1 (which plays a critical role in preventing c-Myc-induced B cell lymphomas) (17, 44) *in vitro*, we next determined if B cell lymphoma cells infected with the WT virus, LMP2A-KO virus, or LMP1/2A-KO virus lost the expression of BIM1. As shown in Fig. 5 (right), both WT virus-infected and LMP2A-KO virus-infected lymphomas contained no cells that costained for both EBNA2 and BIM1, although BIM1 expression was observed in the surrounding uninfected cells (presumably T cells). In the case of the LMP1/2A-KO virus-infected lymphomas, most EBNA2-positive cells likewise contained no detectable BIM1, although a minority of cells that had a lower level of EBNA2 expression expressed BIM1. These results suggest that LMP2A-KO and LMP1/2A-KO virus-induced lymphomas in cord blood-humanized mice may be promoted and sustained by both increased c-Myc expression and decreased BIM1 expression, two properties that are known to be conferred *in vitro* by the EBNA2 and EBNA3A/3C proteins, respectively.

WT, LMP2A-KO, and LMP1/2A-KO virus-infected tumors express similar levels of lytic viral proteins. Whether LMP2A primarily promotes or inhibits lytic EBV

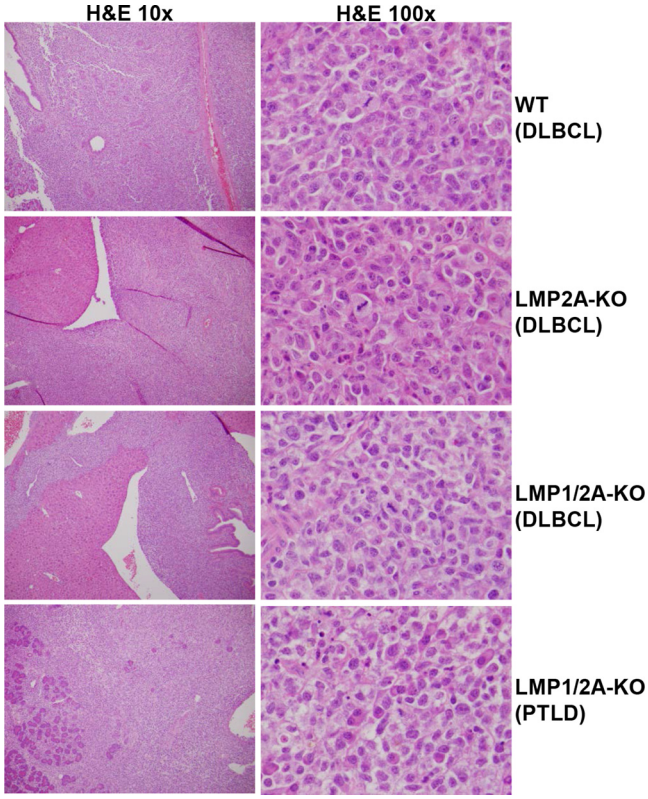

FIG 3 Comparison of the phenotypes of lymphomas induced by the WT, LMP2A-KO, and LMP1/2A-KO viruses. Representative H&E-stained WT EBV-, LMP2A-KO virus-, and LMP1/2A-KO virus-induced tumors (each lymphoma invading the liver or pancreas) are shown. Original magnifications are indicated. All tumors infected with the WT and LMP2A-KO viruses were similar to DLBCLs; the LMP1/2A-KO virus induced both DLBCL-like and polymorphic PTLD-like lesions, as shown.

reactivation in infected human B cells *in vivo* is not totally clear, since LMP2A has been reported to inhibit BCR-mediated lytic viral reactivation of LCLs *in vitro* (45) but to enhance constitutive lytic gene expression when expressed in certain EBV-infected Burkitt lines (46). To determine if constitutive LMP2A expression (with or without concomitant LMP1 expression) in the context of the intact viral genome modulates the level of lytic EBV gene expression in the cord blood-humanized mouse model, we stained tumors with an antibody (Ab) directed against the viral immediate early lytic protein BZLF1. In the context of the B95.8 EBV strain, which we previously showed has very little lytic protein expression in humanized mice (38, 39, 47, 48), the WT-, LMP2A-KO-, and LMP1/2A-KO-induced lymphomas had few, if any, cells expressing BZLF1 (Fig. 6A and C). This result suggests that LMP2A is not required for the inhibition of lytic viral reactivation in the cord blood-humanized mouse model.

Since the M81 strain has been shown to be much more lytic than the B95.8 strain when injected into humanized mice (49), we also examined the expression of two different lytic EBV proteins (BZLF1 and BMRF1) in lymphomas infected by either the WT or LMP2A-deleted M81 strain of EBV. Although we confirmed that WT M81 EBV-infected lymphomas contain numerous lytically infected cells, we did not find that a loss of LMP2A expression in the context of the EBV M81 strain altered the amount of BZLF1 or BMRF1 expression in lymphomas (Fig. 6B, D, and E). Thus, in contrast to previously reported *in vitro* results, endogenous LMP2A expression in the context of the intact viral genome does not appreciably increase, or decrease, the level of lytic EBV infection in the cord blood-humanized mouse model.

LMP2A-KO-, LMP1/2A-KO-, and WT EBV-induced tumors are similarly infiltrated by T cells. We previously showed that T cells infiltrate WT EBV-infected lymphomas in the cord blood-humanized mouse model and demonstrated that immune checkpoint

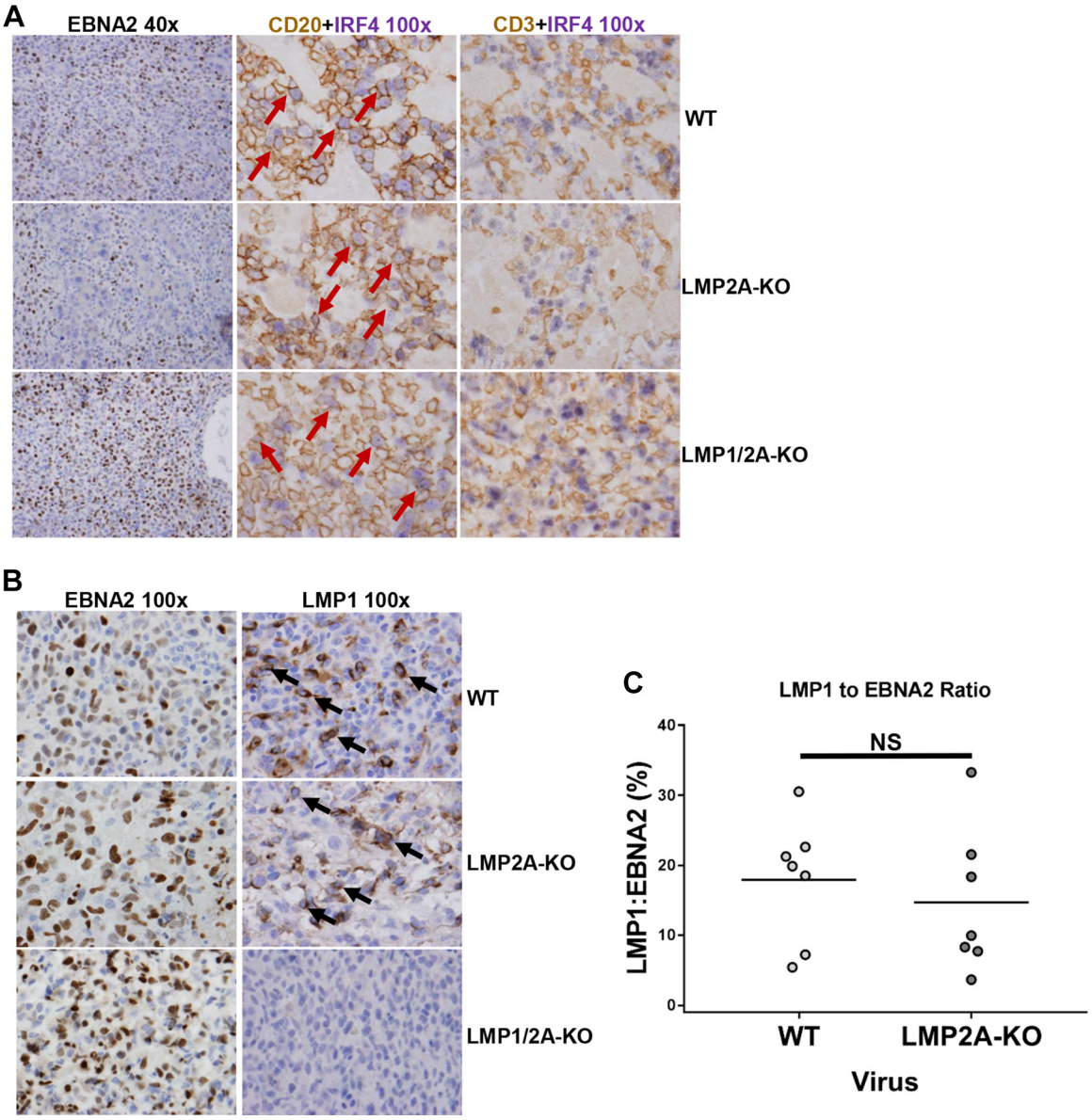

FIG 4 Comparison of IRF4 and LMP1 levels in WT and mutant lymphomas. (A) IHC was performed to detect the EBV latent protein EBNA2 and the cellular proteins IRF4 and CD20, or IRF4 and CD3, as indicated, in WT EBV-, LMP2A-KO virus-, and LMP1/2A-KO virus-induced tumors. EBNA2 single staining is shown, with the original magnification indicated, and IRF4 (purple) and CD20/CD3 (brown) costaining is shown, with the original magnification indicated. Examples of CD20- and IRF4-costained cells are indicated by arrows. (B) EBNA2 and LMP1 expression from WT and LMP2A-KO virus-induced tumors was detected by IHC. The LMP1/2A-KO-induced tumor does not express LMP1. Examples of LMP1-positive cells are indicated by arrows. Original magnifications are indicated. (C) Quantification of the ratio of LMP1-positive to EBNA2-positive cells in 7 different tumor specimens infected by either the WT or the LMP2A-KO virus.

blockade increases the ability of cord blood-derived T cells to infiltrate these lymphomas and slow their growth (39). To determine if the loss of expression of LMP2A and/or the loss of expression of both LMP1 and LMP2A alters the amount of T cell infiltration in EBV-infected lymphomas, lymphomas were stained with antibodies that detect total T cells (CD3), cytotoxic T cells (CD8), or helper T cells (CD4). WT EBV-infected, LMP2A-KO virus-infected, and LMP1/2A-KO virus-infected lymphomas were similarly infiltrated by CD3-positive, CD8-positive, and CD4-positive T cells (Fig. 7). These results suggest that neither LMP2A nor LMP1 grossly alters T cell infiltration of EBV-infected lymphomas in this particular humanized mouse model.

T cells are not required for development of LMP2A-KO virus-induced lymphomas in the cord blood-humanized mouse model. We previously showed that coin-

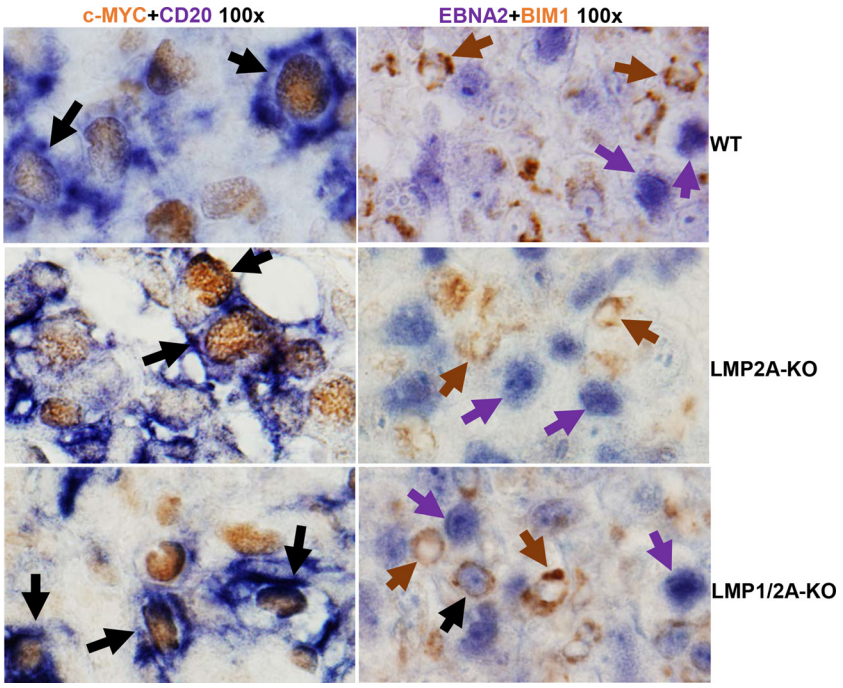

FIG 5 B cells infected with the WT virus, the LMP2A-KO virus, and the LMP1/2A-KO virus express c-Myc but not BIM1. (Left) Representative WT EBV-, LMP2A-KO virus-, and LMP1/2A-KO virus-infected lymphomas were costained for c-Myc (brown) and CD20 (purple). Representative costaining cells are indicated by arrows. The original magnification is indicated. (Right) EBNA2 (blue)-positive, BIM1 (brown)-negative cells are indicated by purple arrows, EBNA2-negative cells expressing BIM1 are indicated by brown arrows, and a cell expressing low levels of EBNA2 and BIM1 is indicated by a black arrow. The original magnification is indicated.

jected CD4 positive T cells are required for the ability of LMP1-deleted EBV, but not wild-type EBV, to cause lymphomas in the cord blood-humanized mouse model and demonstrated that CD40L-producing CD4 T cells can substitute for LMP1 in this model (38). To determine if T cells are required for the development LMP2A-KO-induced lymphomas in this model, mice were injected with LMP2A-KO virus-infected cord blood and then treated with or without a T cell-depleting antibody (OKT3) starting at day 4 after injection. As shown in Fig. 8, the T cell-depleting antibody did not inhibit the ability of the LMP2A-KO virus to establish lymphomas in cord blood-humanized mice, presumably because LMP1 expression substitutes for growth-promoting signals derived from helper CD4 T cells. In contrast, consistent with our previously reported results using the single LMP1-KO virus (38), none of the 10 animals infected with the LMP1/2A-KO virus developed lymphomas when treated with the OKT3 Ab (data not shown).

WT, LMP2A-KO, and LMP1/2A-KO viruses inhibit plasma cell differentiation in the cord blood-humanized mouse model. Finally, given the previously reported ability of EBV to inhibit plasma cell differentiation *in vitro* (31–33), we asked whether EBV infection prevents plasma cell differentiation in the cord blood-humanized mouse model. Splenic B cell follicles from cord blood-humanized mice that were mock infected or infected with the WT, LMP2A-KO, LMP1-KO, or LMP1/2A-KO virus were stained with CD20 antibody, CD138 or BLIMP1 antibodies (plasma cell markers), or (in the case of EBV-infected animals) EBERs (EBV infection markers). As shown in Fig. 9, splenic follicles containing uninfected B cells expressed high levels of CD138 and had occasional BLIMP1-expressing cells, whereas splenic follicles that were composed largely of WT, LMP2A-KO, LMP1-KO (not shown), or LMP1/2A-KO virus-infected B cells expressed little or no CD138 or BLIMP1. These results suggest that EBV infection inhibits plasma cell differentiation in the cord blood-humanized mouse model, even in the absence of LMP1 and LMP2A expression.

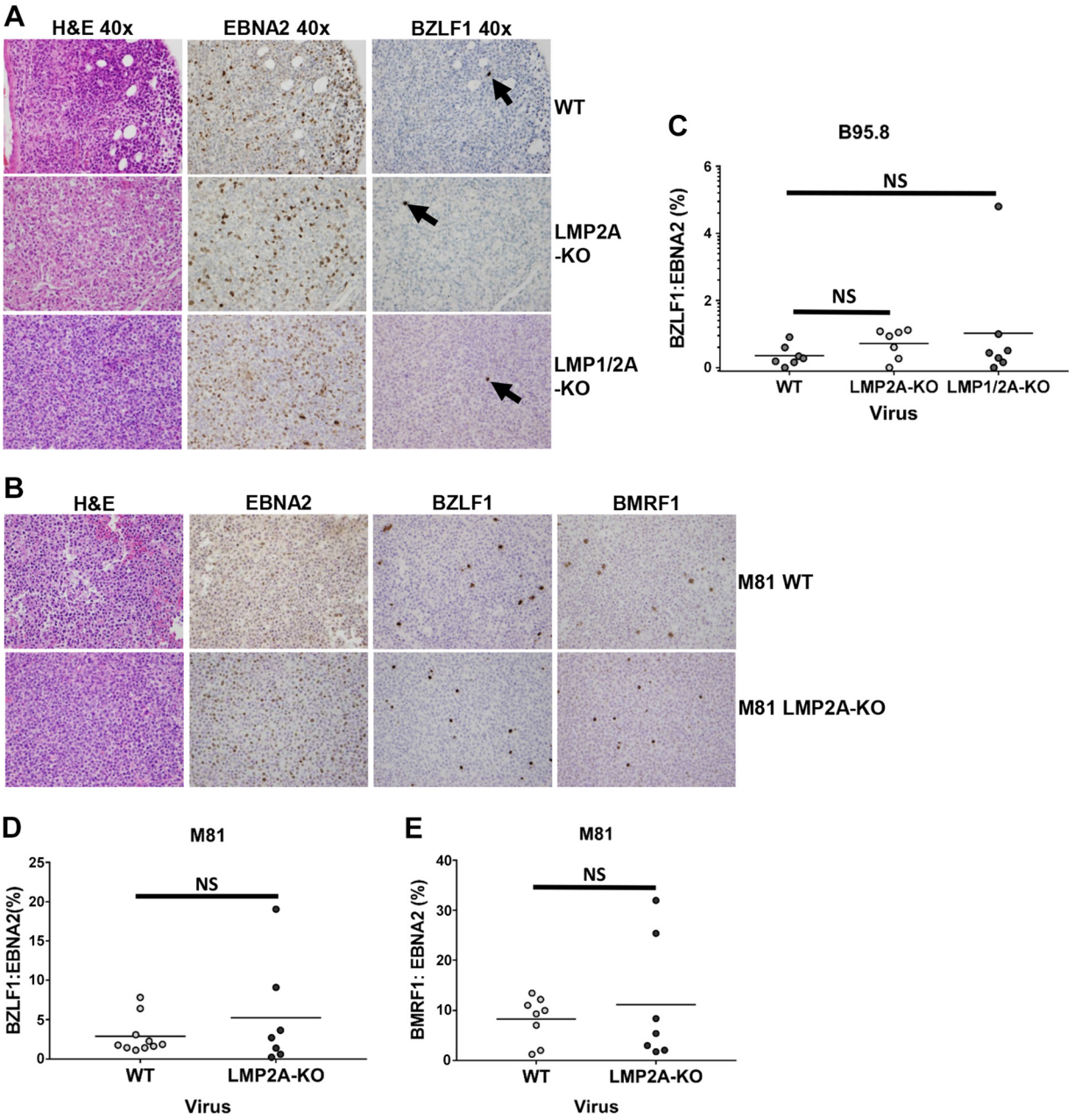

FIG 6 LMP2A-KO-, LMP1/2A-KO-, and WT EBV-induced tumors express similar levels of lytic viral proteins. (A) Tumors infected with B95.8 strain-derived WT, LMP2A-KO, and LMP1/2A-KO viruses were stained with H&E or EBNA2 and BZLF1 antibodies, as indicated. Examples of positively stained cells are indicated with arrows. (B) Tumors infected with the M81 strain-derived WT or LMP2A-KO virus were stained with H&E or EBNA2, BZLF1, and BMRF1 antibodies, as indicated. The original magnification is indicated. (C to E) Quantification of the ratio of Z- or BMRF1- to EBNA2-positive cells in different tumor specimens infected by either the WT or the LMP2A-KO virus (either EBV strain B95.8 or M81, as indicated).

DISCUSSION

In this paper, we used a cord blood-humanized mouse model to examine whether EBV-induced lymphomas in this model require the expression of the EBV LMP2A latency protein. We recently showed that an LMP1-deleted EBV mutant can induce invasive lymphomas in this model, as long as CD4 T cells are present to provide an alternative source of CD40 signaling (38). Since LMP2A mimics the effect of constitutive BCR signaling, and constitutive BCR signaling is commonly selected for in EBV-negative human B cell lymphomas, we expected that the loss of LMP2A expression might inhibit the formation of EBV-induced lymphomas in this model. Instead, we show here that the

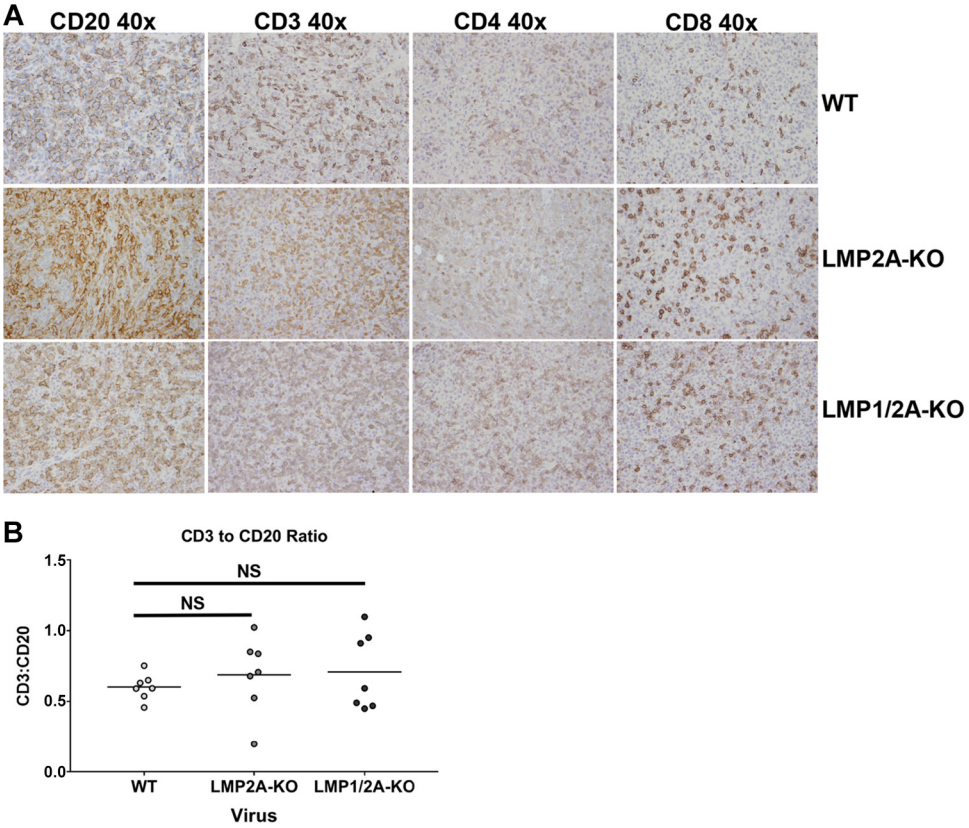

FIG 7 LMP2A-KO-, LMP1/2A-KO-, and WT EBV-induced tumors are similarly infiltrated by T cells. (A) Representative H&E-, CD3-, CD4-, and CD8-stained tumors were infected with WT EBV, LMP2A-KO, and LMP1/2A-KO. The original magnification is indicated. (B) Quantification of the ratio of $CD3^+$ to $CD20^+$ cells in different tumor specimens infected by either the WT, LMP2A-KO, or LMP1/2A-KO virus.

loss of LMP2A alone has little effect on the ability of EBV to form B cell lymphomas, other than increasing the time to tumor onset, and demonstrate that even an EBV mutant simultaneously deleted for both the LMP1 and LMP2A genes still induces invasive lymphomas in a subset of animals. These results suggest that EBV-infected B

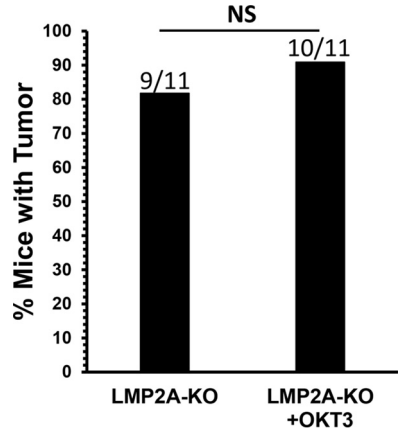

FIG 8 T cells are not required for establishment of LMP2A-KO virus-induced lymphomas in the cord blood-humanized mouse model. LMP2A-KO-infected cord blood-humanized mice were treated with or without the T cell-depleting antibody OKT3, starting 4 days after injection of cells into mice. The proportion of mice with EBV-positive tumors and the numbers of mice with tumors relative to the total number of mice used in each group are shown. The *P* values were calculated by using a two-tailed Fisher exact test.

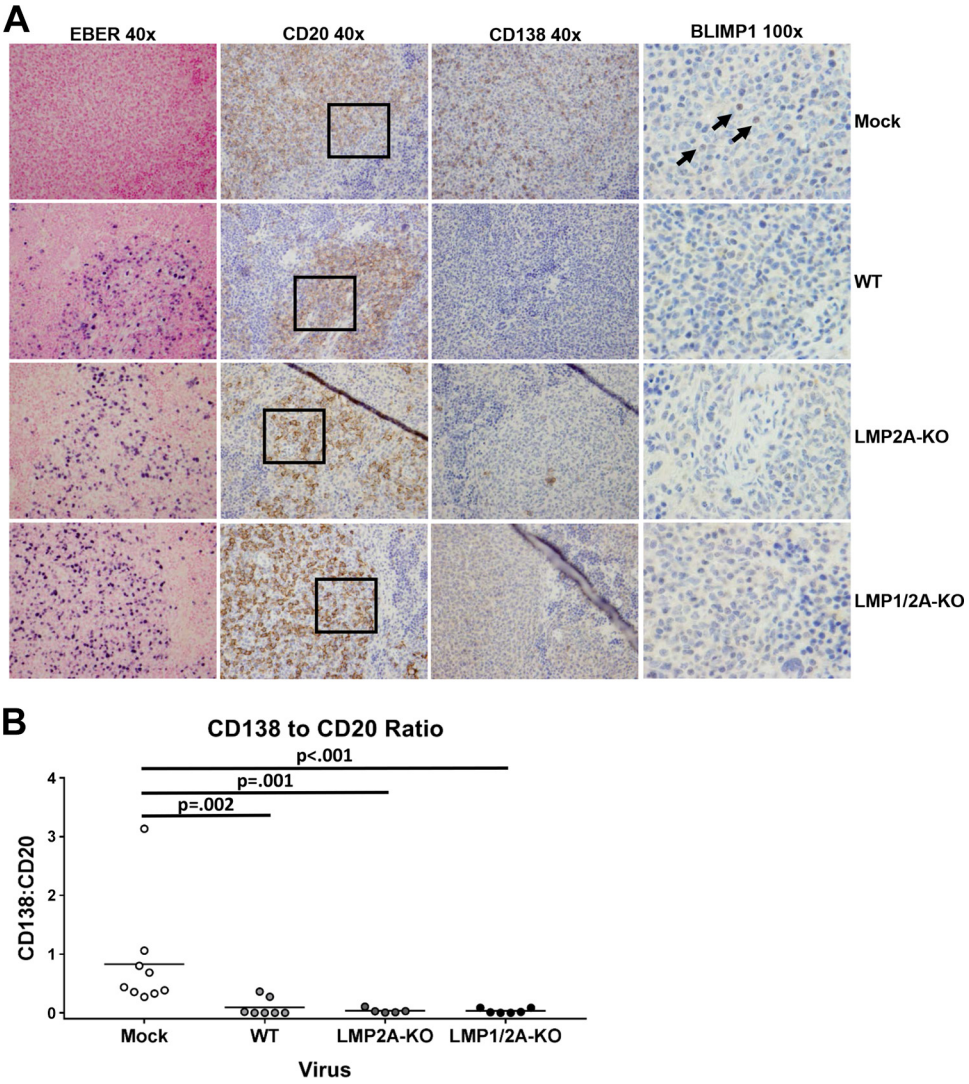

FIG 9 Splenic follicles of WT, LMP2A-KO, and LMP1/2A-KO virus-infected cord blood-humanized mice have less plasma cell differentiation than do splenic follicles of mock-infected mice. (A) Representative EBER-, CD20 Ab-, CD138 Ab-, and BLIMP1 Ab-stained cells in splenic follicles derived from mice infected with the WT, LMP2A-KO, or LMP1/2A-KO virus or mock-infected mice. BLIMP1 stains were done in the boxed area shown in the CD20 stain. CD138 and BLIMP1 are markers of plasma cell differentiation. (B) Quantification of the ratio of $CD138^+$ to $CD20^+$ cells in the splenic follicles of uninfected or infected animals.

cells may obtain growth and/or survival signals *in vivo* that allow the virus to induce invasive DLBCLs in the absence of the expression of both LMP1 and LMP2A. Nevertheless, our finding that the simultaneous deletion of both LMP1 and LMP2A decreases the percentage of animals developing EBV-positive tumors, and substantially increases the time to tumor onset, suggests that the expression of either LMP1 or LMP2A accelerates the onset of virally associated lymphomas and increases the efficiency of this process.

The ability of EBV to transform primary B cells *in vitro* into long-lived LCLs has proven to be an invaluable tool for dissecting mechanisms by which various different EBV proteins might contribute to EBV-associated human lymphomas. Nevertheless, *in vitro* transformation studies may not recapitulate certain critical aspects of EBV-associated malignancies in humans. For example, the supporting roles of the *in vivo* tumor microenvironment, and the various different types of interactions between EBV-infected B cells and T cells, are difficult to model by using *in vitro* systems. Furthermore, although EBV infection of primary B cells *in vitro* initially induces the rapid proliferation of essentially all infected cells (13), most of these infected cells die after a

few weeks via poorly understood mechanisms, and only a small minority of EBV-infected cells actually have the capacity to become long-term LCLs. Whether a similar massive die-off of EBV-infected B cells occurs *in vivo* (in the absence of immune-mediated killing) is not known; if not, then certain viral proteins required to sustain long-term LCLs *in vitro* may not be required for the ability of EBV to form lymphomas *in vivo*. Finally, the ability of EBV-infected B cells to invade organs also cannot be easily studied *in vitro*.

We initiated these studies hoping to develop an *in vivo* system in which the contributions of LMP2A (within the context of the intact viral genome) to the establishment of persistent EBV infection, regulation of EBV lytic reactivation, and/or EBV-induced transformation of B cells might be readily apparent. A number of potential roles for LMP2A in enhancing EBV persistence and/or EBV-associated lymphomas have been proposed, which include regulating the latent-to-lytic switch (45, 46), inducing plasma cell differentiation (35), promoting the survival of B cells that have undergone nonproductive BCR rearrangements (21), cooperating with c-Myc overexpression to induce Burkitt-like lymphomas (50, 51), and inhibiting the host immune response (52, 53). However, many of those previous studies expressed LMP2A at nonphysiological levels and/or were performed outside the context of the intact viral genome. In contrast, our studies here examined the effects of endogenous LMP2A expression in the context of the intact viral genome and in the presence and absence of human T cells.

We did not find that the loss of LMP2A expression alone substantially alters the phenotype of EBV-infected lymphomas in this cord blood-humanized mouse model. Almost all EBV-induced lymphomas in this model, in the presence or absence of LMP1 and/or LMP2A expression, were activated DLBCLs that expressed IRF4 but little if any CD138 (a marker for more differentiated plasma cells). Thus, in contrast to the results of transgenic mouse studies, we did not find that LMP2A enhances plasma cell differentiation in the context of the intact viral genome in this cord blood-humanized mouse model. Of note, however, in contrast to the single LMP1- and LMP2A-deleted lymphomas, a subset of the LMP1/2A-KO double mutant virus-induced lymphomas were more similar to PTLD-like lesions than invasive DLBCLs. These results suggest that the expression of both LMP1 and LMP2A promotes the more invasive DLBCL phenotype in this model.

In addition, in contrast to some previously reported *in vitro* studies, we did not find that endogenous LMP2A expression in the context of the intact viral genome significantly impacts the amount of lytic viral gene expression in the cord blood-humanized mouse model. We examined the effect of the loss of LMP2A on lytic viral protein expression using two different viral strains (the largely latent B95.8 strain and the highly lytic M81 strain), but in both cases, we did not observe any effect on lytic viral protein expression. It remains possible that other viral proteins (such as LMP1 and/or one or more EBNA proteins) have stronger effects on lytic reactivation than LMP2A and that the effect(s) of LMP2A on lytic viral reactivation is restricted to cells that have stringent (type I) viral latency. In addition, EBV-infected B cells in this model may have constitutive BCR signaling in response to foreign tissue antigens, in which case the ability of LMP2A to induce further BCR stimulation might be minimal.

Although LMP2A has been reported to activate survival pathways *in vitro*, including the phosphatidylinositol 3-kinase (PI3K) and NF-κB pathways (54, 55), we did not find that the deletion of LMP2A alone substantially affected the number of EBV-induced lymphomas in this model, although the onset of lymphomas was somewhat delayed. Since LMP1 is also a potent activator of both canonical and noncanonical NF-κB, as well as PI3K (56), it may at least partially substitute for the survival effects of LMP2A. In addition, EBV-infected B cells can be activated by CD40L expressed on CD4 T cells in this model, which would likewise result in NF-κB and PI3K signaling. Although the LMP2A-KO virus induced lymphomas in T cell-depleted animals, as expected, the LMP1/2A-KO virus could not induce lymphomas in T cell-depleted animals. These results suggest that CD40L signaling from CD4-positive T cells is not required to sustain LMP2A-KO-induced tumors unless LMP1 is also deleted.

Although LMP2A has been reported to decrease the expression of major histocompatibility complex (MHC) class II (53) as well as the expression of the NKG2D receptor ligands MICA and ULBP4 (52), the ability of T cells to control WT virus- versus LMP2A-KO virus-induced tumors was not obviously different in the cord blood-humanized mouse model. In both cases, CD4 and CD8 T cells were able to infiltrate the tumors but did not ultimately control them. Although we have recently shown that T cells have some ability to control EBV-induced lymphomas in the cord blood-humanized mouse model, particularly in the presence of immune checkpoint blockade (39), the potential immune-evasive roles of LMP2A might be more clearly observed by using more immunocompetent humanized models containing coengrafted human thymic tissue.

As suggested by several different *in vitro* studies (31–33), we confirm here that EBV infection in the cord blood-humanized mouse model inhibits the ability of B cells to differentiate into plasma cells. Although overexpressed LMP1 has been reported to inhibit plasma cell differentiation *in vitro* by inhibiting BLIMP1 activity (32), we found here that the LMP1/2A-KO virus also inhibited plasma cell differentiation. This result may reflect the recent finding that EBV-encoded BHRF1 microRNAs (which are expected to be expressed in the LMP1/2A-KO mutant) also inhibit plasma cell differentiation *in vitro* (33). It is possible that one or more EBNA proteins also contribute to this phenotype. BLIMP1 is the master regulator of plasma cell differentiation, and BLIMP1-inactivating mutations were recently shown to be very common in activated DLBCLs in humans (57). Thus, the ability of EBV to inhibit terminal plasma cell differentiation likely plays an important oncogenic role in this cord blood-humanized model for EBV-induced DLBCLs. In addition, since plasma cell differentiation induces the lytic form of EBV infection (58), inhibition of plasma cell differentiation may be required to maintain long-term viral latency in humans.

Finally, the fact that the LMP1/2A-KO virus causes highly aggressive and invasive lymphomas in a portion of cord blood-humanized mice potentially makes this an attractive model to dissect the roles of specific EBV EBNA proteins (or EBV-encoded EBERs and/or viral microRNAs) for EBV-induced lymphoma formation *in vivo*. Our findings that lymphomas induced by the LMP1/2A-KO virus have high c-Myc expression levels as well as decreased BIM1 expression (similar to the phenotype of WT-induced tumors) suggest likely mechanisms by which this mutant virus promotes B cell lymphomas *in vivo* and are consistent with the known ability of EBNA2 and EBNA3A/3C, respectively, to activate c-Myc and inhibit BIM1 expression *in vitro*.

MATERIALS AND METHODS

EBVs. Wild-type EBV strains B95.8 (GenBank accession number NC_007605.1) and M81 (GenBank accession number KF373730.1) are available as recombinant bacmids. The LMP1-KO virus (missing amino acids 1 to 384) used in these studies was previously described (59) and was a gift from Wolfgang Hammerschmidt. The single LMP2A-deleted B95.8 strain mutant used in most of these studies (a gift from Wolfgang Hammerschmidt) was created as previously described (18) and deletes the first exon of *LMP2A* (nucleotides [nt] 166170 to 166938 of the genome of the prototype EBV strain B95.8). The M81 strain LMP2A mutant was created by first deleting a region beginning 61 nucleotides before the LMP2A open reading frame and ending 2 nucleotides before the end of LMP2A exon 1 (in B95.8, deletion from nt 166042 to nt 166457; in M81, deletion from nt 165906 to nt 166321). The deletion was achieved by homologous recombination of the recombinant virus with a linear DNA fragment that harbors the kanamycin resistance gene and Flp recombination sites flanked by short DNA regions homologous to LMP2A-specific sequences. The oligonucleotides used were 5′-AGCATCACAGGTTATTTTGCCTGAAGCTTG CTGGGGCGTAAACAGCTATGACCATGATTACGCC-3′ and 5′-TGATGCAATAAATAAAAGTACAGATAGATGGC ACTCTTACCCAGTCACGACGTTGTAAAACGAC-3′. The kanamycin cassette was subsequently excised with the Flp recombinase. The LMP1/LMP2A double-knockout virus used in these studies was created from the second LMP2A deletion described above (in B95.8) by introducing deletions of the first two LMP1 exons and of most of the third LMP1 exon. The deletion reaches from nt 167745 to nt 169148 and was obtained by linear targeting with a PCR product that harbors the tetracycline resistance gene and sequences specific for the LMP1 gene. The oligonucleotides used are 5′-GTCATAGTAGCTTAGCTGAACT GGGCCGTGGGGGTCGTCAGCGTGTTTAGATTGGAGTGAACG-3′ and 5′-TACATAAGCCTCTCACACTGCTCTGC CCCCTTCTTTCCTCGCGGAATAACATCATTTGGTGACG-3′.

Production of infectious virus. Infectious viral particles were produced from 293 cell lines stably infected with the WT or mutant viruses following transfection with EBV BZLF1 and Gp110 expression vectors as previously described (47). The titer of EBV was determined on Raji cells by using the Green Raji cell assay as previously described (47).

TABLE 2 Antibodies used for immunohistochemistry

Antibody	Clone	Manufacturer	Dilution
CD20	H1	BD Pharmingen	1:500
CD20	BV11	Abcam, Inc.	1:100
CD3	Polyclonal	DakoCytomation	1:200
CD4	4B12	Leica Microsystems	1:80
CD8	CRM311	Biocare Medical	1:50
CD138	MI15	DakoCytomation	1:50
LMP1	CS.1-4	DakoCytomation	1:600
EBNA2	PE2	Leica Microsystems	1:100
BZLF1	BZ1	Santa Cruz Biotechnology, Inc.	1:200
BMRF1	G3-E31	Vector Laboratories	1:200
Bim	C34C5	Cell Signaling Technology	1:100
IRF4	MUM1p	Santa Cruz Biotechnology, Inc.	1:50
c-Myc	Y69	Abcam, Inc.	1:50
BLIMP1	Polyclonal	Sigma Life Science	1:500

Humanized NOD/LtSz-*scid/IL2R*γ^{null} mice. Immunodeficient NSG (NOD/LtSz-*scid/IL2R*γ^{null}) mice were purchased from Jackson Laboratory (catalogue number 005557). Commercially purchased CD34-depleted human cord blood mononuclear cells (CB117; AllCells, LLC) were mock infected or infected with WT or mutant viruses *in vitro* for 1.5 h, and 12 million to 25 million cells were then injected i.p. into 3- to 5-week-old NSG mice. Experiments using B95.8-derived bacmids were done by using 10,000 infectious viral particles (titers were determined by the Green Raji cell assay); experiments using bacmids derived from the M81 strain virus were done by using 2,000 infectious units. Mice were sacrificed at day 70 postinfection unless they developed symptoms requiring euthanasia prior to day 70. Both WT and mutant virus-infected animals were included in many of the cord blood experiments so that results could be compared by using the same sets of cord blood. In some experiments, animals were treated with the T cell-depleting OKT3 antibody (50 μg of antibody three times per week i.p.) starting 4 days after EBV infection and continuing for the remainder of the experiment.

Analysis of EBV infection and tumors. Following euthanasia, multiple different organs (including the lungs, spleen, pancreas, liver, gallbladder, mesenteric fat, and abdominal lymph nodes) were formalin fixed and then examined by using a variety of techniques to determine if animals had persistent EBV infection and/or EBV-positive lymphomas and to assess the viral protein expression pattern. Samples from all EBV-infected animals (infected with either the WT virus, the LMP2A-KO virus, or the LMP1/2A-KO virus) were examined by H&E staining to determine if tumors were present and to assess the types of tumors in each animal. Tumors from at least 7 different animals infected with the WT virus, the LMP2A-KO virus, or the LMP1/2A-KO virus (B95.8 strain) also underwent IHC staining by using the antibodies listed in Table 2, as previously described (47, 48). IHC staining was also performed on tumors from at least 4 different animals infected with the WT or LMP2A-KO virus in the context of the M81 strain of EBV. Coauthor Erik A. Ranheim, a board-certified hematopathologist, performed the pathological analysis of the tumors described in Table 1. In some animals, EBER *in situ* hybridization studies were performed by using the PNA ISH detection kit (DakoCytomation) as previously described (47). For quantification of IHC results, at least 2 random fields of view were selected per animal, photographed, and then counted by 2 independent observers. The counts of positively staining cells were averaged across observers. For each ratio (i.e., CD3 to CD20), stains were done on adjacent slides, and counts were completed on the same field of view.

Analysis of EBV mutations in tumors. To confirm that tumors derived from EBV mutants contained the expected deletions, in some animals, DNA was isolated from paraffin-fixed slides by using the QIAamp DNA FFPE (formalin-fixed paraffin-embedded) tissue kit and then PCR amplified by using primers specific for the EBV LMP2A gene (left, CCCTAGAAATGGTGCCAATG; right, ATGAGTCATCCCGTG GAGAG [315 bp]), the LMP1 gene (left, AGTCATCGTGGTGGTGTTCA; right, TTACCACACCCCCACTTTTC [291 bp]), or the BZLF1 gene promoter (left, ACCAGCCTCCTCTGTGATGT; right, TTTGGACGAACTGACC ACAA [298 bp]) to confirm that LMP2A-KO- or LMP1/2A-KO-induced tumors were not contaminated by WT EBV. PCR was performed in a 50-μl reaction mixture volume containing 0.2 μmol/liter primers and 1 U *Taq* DNA polymerase, under the following conditions: 95°C for 2 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s; and 72°C for 5 min. PCR products were visualized with ethidium bromide on a 1% agarose gel.

Statistics. Mstat software (http://mcardle.wisc.edu/mstat/download/index.html) was used to statistically analyze tumor incidence and B cell engraftment data at study completion. Two-tailed Fisher's exact test was used to compare tumor formation and B cell engraftment between different viruses. Mann-Whitney tests were performed on cell ratios for different viruses by using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). A Kaplan-Meier curve was employed (GraphPad Prism) to determine differences in rates of tumor development postinjection by using a log rank test. Animals that did not develop tumors were censored at study completion. Differences were considered significant at a P value of <0.05.

Study approval. All animal work experiments were approved by the University of Wisconsin—Madison Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (60).

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